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Retinoic Acid Induces Neurogenesis by Activating Both Retinoic Acid Receptors (RARs) and Peroxisome Proliferator-activated Receptor β/δ (PPARβ/δ)*

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Background: RA regulates transcription through the CRABP-II/RAR and FABP5/PPARβ/δ pathways, but the contributions of these pathways to RA-induced neuronal differentiation are unknown.
Results: RA signaling switches from CRABP-II/RAR to FABP5/PPARβ/δ during differentiation. The switch is controlled by transient up-regulation of RARβ and the CRABP-II/FABP5 ratio.
Conclusion: Both RA pathways are employed during neuronal differentiation.
Significance: The data contribute insights into RA-induced neurogenesis.

Retinoic acid (RA) regulates gene transcription by activating the nuclear receptors retinoic acid receptor (RAR) and peroxisome proliferator-activated receptor (PPAR) β/δ and their respective cognate lipid-binding proteins CRABP-II and FABP5. RA induces neuronal differentiation, but the contributions of the two transcriptional pathways of the hormone to the process are unknown. Here, we show that the RA-induced commitment of P19 stem cells to neuronal progenitors is mediated by the CRABP-II/RAR path and that the FABP5/PPARβ/δ path can inhibit the process through induction of the RAR repressors SIRT1 and Ajuba. In contrast with its inhibitory activity in the early steps of neurogenesis, the FABP5/PPARβ/δ path promotes differentiation of neuronal progenitors to mature neurons, an activity mediated in part by the PPARβ/δ target gene *PDK1*. Hence, RA-induced neuronal differentiation is mediated through RAR in the early stages and through PPARβ/δ in the late stages of the process. The switch in RA signaling is accomplished by a transient up-regulation of RARβ concomitantly with a transient increase in the CRABP-II/FABP5 ratio at early stages of differentiation. In accordance with these conclusions, hippocampi of FABP5-null mice display excess accumulation of neuronal progenitor cells and a deficit in mature neurons *versus* wild-type animals.

All-*trans*-retinoic acid (RA)[2] plays important roles in central nervous system development (1) and has been widely used to study neuronal differentiation of cultured embryonic stem cells. The pluripotent P19 mouse embryonal carcinoma cell line has been an especially useful model for studying RA-induced neuronal differentiation. Treatment of these cells with RA results in the formation of embryonic bodies resembling the blastula stage. Re-plating of these cell aggregates then gives rise to neuronal and glial cells (2, 3). The biological activities of RA originate from its ability to activate several members of the nuclear receptor family of transcription factors as follows: the classical RA receptors RARα, RARβ, and RARγ (4) and the peroxisome proliferator-activated receptor β/δ (PPARβ/δ) (5–9). The partitioning of the hormone between its receptors is regulated by two intracellular lipid-binding proteins that deliver it from sites of synthesis in the cytosol to cognate receptors in the nucleus; cellular RA-binding protein II (CRABP-II) transports RA to RARs, and fatty acid-binding protein type 5 (FABP5) shuttles the ligand to PPARβ/δ. Hence, the spectrum of target genes activated by RA and the biological responses to the hormone are determined by the relative expression of these binding proteins in specific cells; RA controls expression of RAR target genes in cells that display a high CRABP-II/FABP5 ratio, but it regulates PPARβ/δ target genes in cells in which this ratio is low (7, 8, 10–14). An additional RA-binding protein that may be involved in regulating the activities of the hormone is CRABP-I. It was thus reported that CRABP-I dampens the response of F9 teratocarcinoma cells to RA and that it does so by enhancing the degradation of the hormone (15, 16).

It is well established that activation of RAR by RA is essential for induction of neuronal differentiation, and various RAR target genes were reported to be involved in the process (1, 17). These include primary targets, *e.g. Hoxa-1, Hoxb-2, Sox6,* and *Wnt-1*, and indirect targets such as *Mash-1, Ngn-1, NeuroD, N-cadherin,* and *Pbx*. Components of the alternative RA pathway, FABP5 and PPARβ/δ, are highly expressed in embryonic brain. In rat brain, both proteins appear at mid-term, around day E10.5. FABP5 expression peaks at birth and gradually decreases to attain a lower sustained expression in adult brain. PPARβ/δ expression peaks at E13.5–15.5 days and then decreases slightly but remains high through development and adult life (18, 19). It was reported that activation of PPARβ/δ

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[2] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; PPAR, peroxisome proliferator-activated receptor; Q-PCR, quantitative real time PCR; RXR, retinoid X receptor; TTNPB, 4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid.

induces oligodendrocyte differentiation and enhances neuronal maturation in cultured cell models of neurogenesis (20, 21) and that, by exerting anti-apoptotic and anti-inflammatory functions, PPARβ/δ displays neuroprotective activities (22). Available information thus raises the possibility that both RAR and PPARβ/δ are involved in mediating RA-induced neuronal differentiation. However, the contributions of the two paths to the overall process are poorly understood.

Here, we show that the CRABP-II/RAR path mediates the ability of RA to induce commitment of stem cells to neuronal progenitors and that the FABP5/PPARβ/δ pathway is critical for subsequent progenitor cell differentiation into mature neurons. The shift in RA signaling during neurogenesis is accomplished by a transient increase in the CRABP-II/FABP5 ratio in stem cells undergoing differentiation to neuronal progenitors. In accordance with the conclusion that the RA-activated FABP5/PPARβ/δ pathway is critical for promoting differentiation of neuronal progenitor cells to mature neurons, the hippocampus of FABP5-null mice displays an excess of neuronal progenitor cells and a deficit in mature neurons.

EXPERIMENTAL PROCEDURES

Reagents—Goat anti-mouse FABP5 polyclonal antibodies were obtained from R&D Systems. Antibodies against PPARβ/δ (H-74 for ChiP assays), pan-RXR, Oct3/4, and actin (I-19) were obtained from Santa Cruz Biotechnology. Antibodies against nestin, MAP2, NeuN, SIRT1, and PPARβ/δ were from Millipore. Antibodies against β3-tubulin and glial fibrillary acidic protein were from Abcam. Antibodies for Ajuba and PDK1 were from Cell Signaling and BD Transduction Laboratories, respectively. Antibodies against CRABP-II were a gift from Cecile Rochette-Egly (Institut Génétique Biologie Moléculaire Cellulaire, Illkirch, France). The HRP-conjugated secondary antibodies were obtained from Sigma or Bio-Rad. Alexa Fluor 488- and 555-conjugated secondary antibodies were from Invitrogen. GW0742 and 4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) were obtained from Toronto Research Diagnostics and Biomol International, Inc., respectively. Retinoic acid, retinol, and retinyl acetate were obtained from Sigma.

Constructs—Mouse FABP5 and PPARβ/δ ORFs were cloned into the mammalian expression vector pLVX-IRES-ZsGreen1 (Clontech). Expression vectors for Ajuba (Clone ID 4187633) and PDK1 (Clone ID 5176294) were obtained from Open Biosystems. The mouse SIRT1 expression construct of pCruzHA-mSIRT1 was obtained from Addgene. The lentiviral vectors that harbored mouse FABP5 short hairpin RNA (shRNA: TRCN0000011894) and mouse PPARβ/δshRNA (TRCN0000026045) were obtained from Open Biosystems.

Cell Culture and Differentiation—P19 cells were obtained from ATCC. All experiments were performed with cells within 10 passages. Transfections were carried out using Lipofectamine 2000 (Invitrogen). Cells stably expressing shRNAs were selected using puromycin (2.5 μg/ml).

P19 cell were induced to undergo neuronal differentiation according to established procedures (23). Briefly, cells were cultured on 1% agarose-coated 10-cm dishes at 3.0×10^5 cells/ml in α-minimal essential medium (Hyclone) supplemented with 10% FBS. Differentiation was induced by addition of RA (1 μM) and medium containing RA replaced 2 days later. On day 4, cell aggregates were collected by centrifugation, separated to single cells by trypsin/EDTA treatment, replated onto poly-L-lysine-coated plates, and cultured in α-minimal essential medium supplemented with 10% FBS. On day 6, medium was replaced with neurobasal medium containing B27 supplement (Invitrogen) and 2 mM GlutaMAX. Medium was replaced every 2 days for an additional week.

Quantitative Real Time PCR (Q-PCR)—RNA was isolated by using TRIzol (Molecular Research Center, Inc.). cDNA was generated by using the High Capacity cDNA reverse transcription kit (Applied Biosystems). Q-PCR was carried out using TaqMan Gene expression master mix and probes (Applied Biosystems) as follows: CRABP-II (Mm00801691_m1); FABP5 (Mm00783731_s1); PPARβ/δ (Mm01305434_m1); RARα (Mm00436264_m1); RARβ (Mm01319680_m1); RARγ (Mm00441083_m1); nestin (Mm01223403_m1); Sox2 (Mm03053810_s1); SIRT1 (Mm01168521_m1), Ajuba (Mm00495049_m1); PDK1 (Mm00440707_m1); Adfp (also known as Perlipin 2) (Mm00475794_m1); c-Myc (Mm00487803_m1); 18 S rRNA (4352930E), and GAPDH (Mm99999915_g1).

Chromatin Immunoprecipitation (ChiP) Assays—Proteins were cross-linked to DNA by adding 1% formaldehyde (10 min., 37 °C). The reaction was quenched with 0.125 M glycine, and cells were rinsed with cold PBS, scraped, and collected. Cell pellets were resuspended in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitors) and centrifuged. Pellets were lysed in nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors). Chromatin was sonicated to ~300–3000 bp, and the debris was removed by centrifugation and the supernatant diluted in IP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, and 1.1% Triton X-100) at 1:10 ratio. Samples were precleared with protein A beads/salmon sperm/tRNA mixture (1 h, 4 °C) and incubated with the appropriate antibodies (4 °C, overnight). Protein A beads/salmon sperm/tRNA mixture was added (2 h, 4 °C), and beads were collected by centrifugation and consecutively washed with IP dilution buffer, TSE-500 buffer (20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100), LiCl/detergent wash buffer (100 mM Tris-HCl, pH 8.1, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid), and TE buffer. Complexes were eluted with elution buffer (100 mM $NaHCO_3$, 1% SDS), and cross-linking was reversed (65 °C, overnight). Proteins and RNA were digested with proteinase K and RNase, and DNA was extracted using phenol/chloroform and precipitated with 100% ethanol. The regions that contained putative PPAR-response elements regions were amplified by PCR using the following primers: *Ajuba* forward 5′-GCA TCA CAA GCA GGG AAC TGT GGA-3′ and reverse 5′-CCT CCG CGC ATT CCA ACT GTA AAC-3′; *Sirt1 PPRE1* forward 5′-GAG AAC CAA CCA CTC TCC CTT CTG-3′ and reverse 5′-CTC CCA ATC TCC CTT CTC AAG TGC-3′; *Sirt1 PPRE2* forward 5′-TAG GCA GTT GAT GGT GGC ACA GG-3′ and reverse 5′-TCA AAC CTA GGG CCT CTG CAG GA-3′; *PPARβ/δ-PPRE1* forward 5′-CTG TCC GTC CAT CCG TCT GTC TGT-3′ and reverse 5′-CAG GTA AAG GCA CCA GCT GCC

AAG-3′; and *PPARβ/δ-PPRE2* forward 5′-CCT GTA CTG GCT CTA GAA TGT TTG C-3′ and reverse 5′-TCA TCA TAC.

Mouse Studies—WT and FABP5-null C57BL/6 littermate mice were maintained on a 12-h light and dark cycle on a normal chow diet. Mice were housed according to ARC protocol. The breeding diet (5P76 from LabDiet) contained 25,000–29,000 IU of vitamin A per kg. Mice had access to water and diet *ad libitum*.

Immunocytofluorescence—Cells were washed with PBS, fixed in 4% paraformaldehyde/PBS, blocked, and permeabilized with PBS containing 0.3% Triton X-100, 1% BSA (30 min, 25 °C) and were incubated with primary antibodies (4 °C, overnight). Cells were washed and stained using fluorescent secondary antibody (25 °C, 1 h) and then by DAPI (2 min). Cells were mounted with Fluoromount-G (SouthernBiotech) and imaged using a LSM510 confocal micrpscope (Leica).

Histology—12–14-Week male mice were perfused with 4% paraformaldehyde/PBS. Brains were extracted, fixed in 4% paraformaldehyde/PBS for 2–4 h, rinsed with PBS, and transferred into 30% sucrose/PBS. Brains were cut half in half, embedded with ODC freezing medium, and frozen in −45 °C isopentane for 10 s. Samples were wrapped in foil and stored at −80 °C. Brains were cut into 20-μm sections using a microtome, placed on slides, and air-dried (25 °C, overnight). Slides were stored at −80 °C until used. For immunostaining, slides were thawed (37 °C, overnight), placed in PBS supplemented with 0.1% Tween 20 (10 min), washed, and placed in PBS containing 10% normal goat or donkey serum and 0.3% Triton X-100 (1–3 h, 4 °C). Primary antibodies were added (4 °C, overnight), washed, and incubated with fluorescent secondary antibodies (45 min, room temperature). Slides were rinsed, incubated with DAPI (5 min), and mounted with Fluoromount-G. Staining and imaging were performed using an LSM510 confocal microscope (Leica).

RESULTS

Neuronal Differentiation Is Accompanied by a Transient Shift in RA Signaling toward the CRABP-II/RAR Pathway—The involvement of RA and its binding proteins and nuclear receptors in neurogenesis was examined using P19 cells, a well established cultured cell model of neuronal differentiation (2, 3, 24). P19 cells were cultured in agarose-coated plates and induced to differentiate by treatment with RA. Four days post-induction, cell aggregates were collected, dissociated, and cultured in poly-L-lysine-coated plates in medium containing 10% FBS but not supplemented with RA. On day 6, the medium was changed to neurobasal medium containing B27 supplement and GlutaMAX (see "Experimental Procedures"). The progress of the differentiation program was evident by loss of the stem cell marker Oct3/4 at day 2, transient up-regulation of the neural progenitor marker nestin, whose mRNA level peaked at day 6, appearance of the immature neuronal marker β3-tubulin at day 4, and finally, appearance of the mature neuronal markers MAP2 and NeuN on days 6–12 (Fig. 1, *a* and *b*).

Undifferentiated P19 cells express the RA receptors RARα, RARβ, RARγ, and PPARβ/δ, as well as the RA-binding proteins CRABP-II and FABP5 (Fig. 1*c*). Induction of differentiation by treatment of cells with RA resulted in transient up-regulation of CRABP-II and down-regulation of FABP5 that were observed at the level of both the respective proteins (Fig. 1*d*) and mRNAs (Fig. 1, *e–g*). Following the initial decrease, the level of both FABP5 protein (Fig. 1*d*) and mRNA (Fig. 1*f*) increased to attain a 2–2.5-fold higher level in mature neurons as compared with undifferentiated P19 cells. Induction of differentiation did not markedly affect the levels of either RARα or PPARβ/δ. The level of RARγ mRNA decreased by about 5-fold by day 4 and remained low in mature neurons (data not shown). Interestingly, concomitantly with the transient up-regulation in expression of CRABP-II, induction of differentiation resulted in up-regulation of RARβ mRNA whose expression peaked at day 4 and subsequently decreased (Fig. 1*h*).

The observations that expression of both CRABP-II and its cognate receptor RARβ is up-regulated early during the neuronal differentiation process suggest that initial events in the process are driven by RA through the CRABP-II/RAR path. In agreement with this notion, the synthetic RAR agonist TTNPB, but not the PPARβ/δ agonist GW0742, mimicked the ability of RA to increase the expression of CRABP-II and decrease the expression of FABP5 (Fig. 2, *a* and *b*). Treatment with TTNPB also induced the expression of the neuronal progenitor markers nestin and β3-tubulin (Fig. 2, *c* and *d*) and decreased the expression of the stem cell marker Oct3/4 (Fig. 2*d*).

RA Signaling through the FABP5/PPARβ/δ Path Inhibits the Formation of Neuronal Progenitors but Enhances Progenitor Maturation—The shift in RA signaling away from the FABP5/PPARβ/δ path in the early stages of neuronal differentiation may signify that the path is detrimental for the process. The effects of varying the expression of FABP5 and PPARβ/δ on the formation of neuronal progenitors were thus investigated. P19 cells in which the expression of FABP5 and PPARβ/δ is stably reduced using corresponding shRNAs were generated (Fig. 3, *a* and *b*). Interestingly, down-regulation of FABP5 also reduced the expression of PPARβ/δ, suggesting that the receptor is subject to auto-regulation by the FABP5/PPARβ/δ path. Cells were treated with RA, and their differentiation to neural progenitors was followed by monitoring the expression of nestin. Nestin expression reached a higher level in cells with decreased expression of either FABP5 or PPARβ/δ (Fig. 3, *c* and *d*). Conversely, ectopic overexpression of either PPARβ/δ or FABP5 (Fig. 3*e*) decreased the expression of nestin (Fig. 3*f*). Notably, as transfection efficiency in these experiments, assessed by fluorescence microscopy, was ~30% (data not shown), the observed changes in nestin expression upon varying the expression of FABP5 and PPARβ/δ underestimates the magnitude of the effect. The data thus suggest that the FABP5/PPARβ/δ path inhibits the differentiation of stem cells to neuronal progenitors.

Surprisingly, immunohistochemistry showed that although cells with decreased expression of FABP5 or PPARβ/δ formed nestin-expressing neuronal progenitors more readily, they displayed significantly lower expression of the later neuronal markers β3-tubulin (Fig. 4*a*). Immunoblot analysis showed further that down-regulation of either FABP5 or PPARβ/δ resulted in decreased expression of both β3-tubulin (Fig. 4*b*) and the mature neuronal marker NeuN (Fig. 4*c*). These observations suggest that the FABP5/

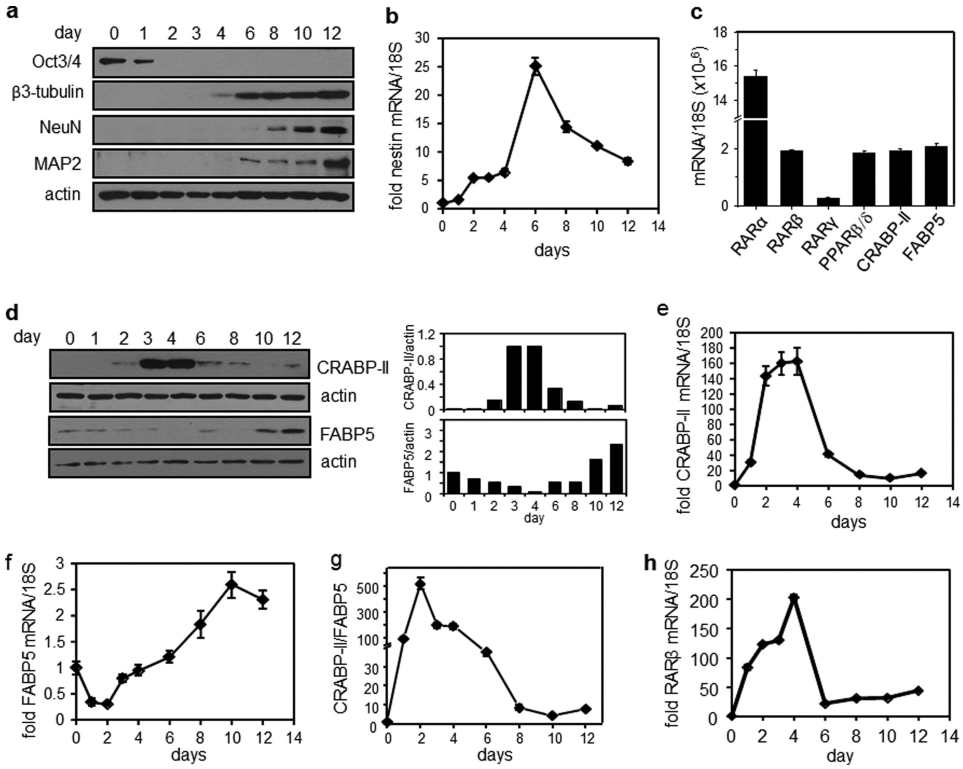

FIGURE 1. **CRABP-II/FABP5 ratio shifts during RA-induced neuronal differentiation.** P19 cells were induced to differentiate as described under "Experimental Procedures." RA was added on day 0. *a,* immunoblots demonstrating expression of Oct3/4, β3-tubulin, NeuN, and MAP2 during differentiation. *b,* levels of nestin mRNA during differentiation were assessed by Q-PCR. *c,* levels of mRNAs for denoted genes in predifferentiated P19 cells were measured by Q-PCR and normalized to 18 S rRNA. Data are mean ± S.D. of three biological replicates. *d, left panel,* immunoblots showing levels of CRABP-II and FABP5 during differentiation. *Right panel,* quantitation of immunoblots showing mean band intensities from two independent experiments. *e* and *f,* levels of mRNA for CRABP-II (*d*) and FABP5 (*e*) during differentiation were assessed by Q-PCR and normalized to respective levels in pre-differentiated cells. Data are means ± S.D. of three independent experiments. *g,* ratio of CRABP-II/FABP5 mRNAs during differentiation, measured by Q-PCR. *h,* levels of RARβ mRNA during differentiation were assessed by Q-PCR and normalized to level in pre-differentiated cells. Data are means ± S.D. of three independent experiments.

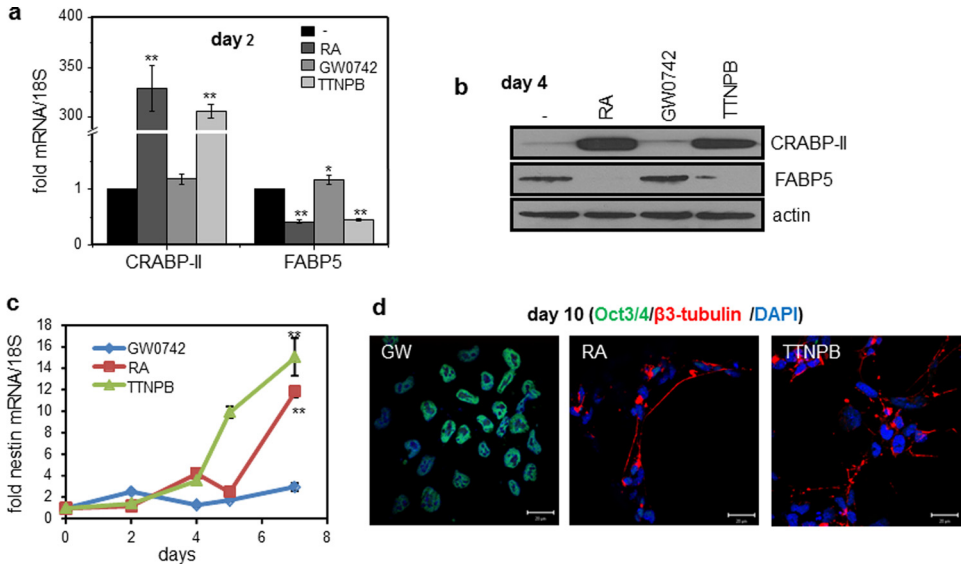

FIGURE 2. **RA triggers neuronal differentiation by activating RAR.** P19 cells were treated with RA, the PPARβ/δ agonist GW0742, or the RAR agonist TTNPB (1 μM), and differentiation was followed as described under "Experimental Procedures." *a,* expression levels of CRABP-II and FABP5 mRNA were assessed by Q-PCR 2 days following induction of differentiation. Data are means ± S.D. of three independent experiments. *, $p < 0.01$; **, $p < 0.001$ *versus* nontreated controls. *b,* expression levels of CRABP-II and FABP5 were assessed by immunoblots at day 4 following treatment with RA, GW0742, or TTNPB. *c,* levels of nestin mRNA were measured by Q-PCR. Data are means ± S.D., $n = 3$. **, $p < 0.001$ *versus* GW0742-treated controls. *d,* cells were fixed at day 10 and stained using DAPI to visualize nuclei (*blue*) and with antibodies against Oct3/4 (*green*) and β3-tubulin (*red*). Images were obtained using confocal fluorescence microscopy. *Bar,* 20 μm.

PPARβ/δ path, which inhibits early events in the differentiation process, may be necessary for enabling neuronal maturation.

Notably, although it is well established that RA triggers neuronal differentiation, it is not clear whether the presence of the hormone is necessary throughout the differentiation program.

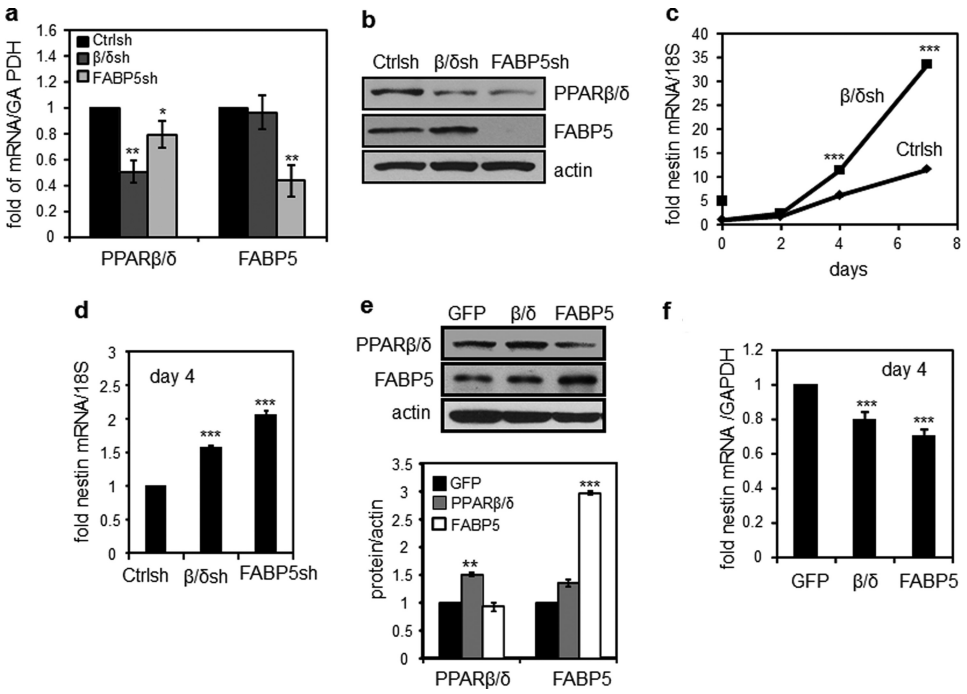

FIGURE 3. **PPARβ/δ/FABP5 pathway inhibits RA-induced differentiation of P19 cells to neuronal progenitors.** *a* and *b,* P19 cell lines with reduced expression of PPARβ/δ or FABP5 were established by stable transfection of corresponding shRNA. Scrambled shRNA was used as control. Down-regulation of PPARβ/δ and FABP5 expression was confirmed by Q-PCR (*a,* mean ± S.D. of three plates; *, $p < 0.05$; **, $p < 0.01$) and immunoblots (*b*). *c* and *d,* cells expressing scrambled shRNA (*Ctrlsh*) or denoted shRNAs were induced to differentiate by addition of RA and levels of nestin mRNA measured by Q-PCR. Data are mean ± S.D. of three independent experiments. ***, $p < 0.001$ *versus* control shRNA. *e,* P19 cells were transfected with vectors harboring GFP, PPARβ/δ, or FABP5 cDNAs. The empty vector pLVX-IRES-GFP was used as control. *Top panel,* overexpression was assessed 48 h post-transfection by immunoblots. *Bottom panel,* quantitation of band intensities from three plates. Data are means ± S.D.; **, $p < 0.01$; ***, $p < 0.001$ *versus* GFP-expressing cells. *f,* cells ectopically overexpressing GFP, PPARβ/δ, or FABP5 were induced to differentiate by addition of RA, and nestin expression was measured by Q-PCR at day 4. Data are mean ± S.D. of three independent experiments. ***, $p < 0.001$ *versus* GFP control.

Standard protocols prescribe removal of RA on day 4 but retain vitamin A in cell media throughout differentiation. Specifically, following removal of RA, cells are usually cultured in medium supplemented with 10% FBS, which contains retinol, followed by culturing in neurobasal medium with B27 supplement that includes 0.35 μM retinol and 0.3 μM retinyl acetate (Invitrogen). To examine whether retinoids are necessary throughout the differentiation process, cells were cultured in retinoid-depleted (charcoal-treated) medium post day 4. Depletion of vitamin A suppressed the appearance of MAP2 on day 12 (Fig. 4, *d* and *e*) and supplementation of media with either retinol and retinyl acetate at concentrations corresponding to the standard B27 supplement or with RA reversed the inhibition and recovered MAP2 expression (Fig. 4, *d* and *e*). Similarly to RA, supplementation of retinoid-depleted media with the PPARβ/δ ligand GW0742 also recovered MAP2 expression (Fig. 4, *d* and *e*). The data thus show that RA is necessary throughout the differentiation program and suggest that activities of the hormone in late stages of differentiation are mediated by PPARβ/δ.

Neurogenesis Inhibitors SIRT1 and Ajuba Are Direct PPARβ/δ Target Genes—To gain insight into the mechanism by which FABP5 and PPARβ/δ suppress the ability of RA to support early events in neuronal differentiation, we considered two proteins that were reported to inhibit the transcriptional activity of RAR in neuronal precursors. One of these is SIRT1, an NAD^+-dependent class III histone deacetylase that was shown to inhibit RA-induced differentiation of P19 cells by competing with RAR for the coactivator SKI-interacting protein (25). The other is Ajuba, a member of the Ajuba/Zyxin family of LIM proteins (26), which was shown to inhibit RA-induced P19 cell differentiation by interacting with RARα to repress its transcriptional activity (27). In agreement with these reports, ectopic overexpression of either SIRT1 or Ajuba (Fig. 5*a*) decreased the expression of nestin at day 4 post-induction of differentiation (Fig. 5*b*). Note again that, due to low transfection efficiency in these experiments, the data underestimate the magnitude of the effects.

RA induced the expression of both Ajuba and SIRT1 (Fig. 5, *c* and *d*). RA also induced the expression of the PPARβ/δ target gene ADFP (28) and the RAR target gene RARβ and of PPARβ/δ itself (Fig. 5*d*). The increase in the level of PPARβ/δ mRNA was modest but functionally meaningful as demonstrated by the observations that it was accompanied by a significant increase in PPARβ/δ protein (Fig. 1*c*). Similarly to RA, the synthetic PPARβ/δ-selective agonist GW0742 also induced the expression of Ajuba, SIRT1, ADFP, and PPARβ/δ (Fig. 5*d*, *inset*). Hence, the data indicate that RA can signal through both of its pathways in undifferentiated P19 cells and support the existence of a positive feedback loop through which PPARβ/δ up-regulates its own expression.

Pretreatment of cells with the protein synthesis inhibitor cycloheximide did not block the ability of RA to up-regulate the expression of ADFP, Ajuba, SIRT1, or PPARβ/δ (Fig. 5*d*). Notably, treatment with cycloheximide alone up-regulated the expression of Ajuba and SIRT1. The ability of cycloheximide to increase the levels of some mRNAs has been previous reported

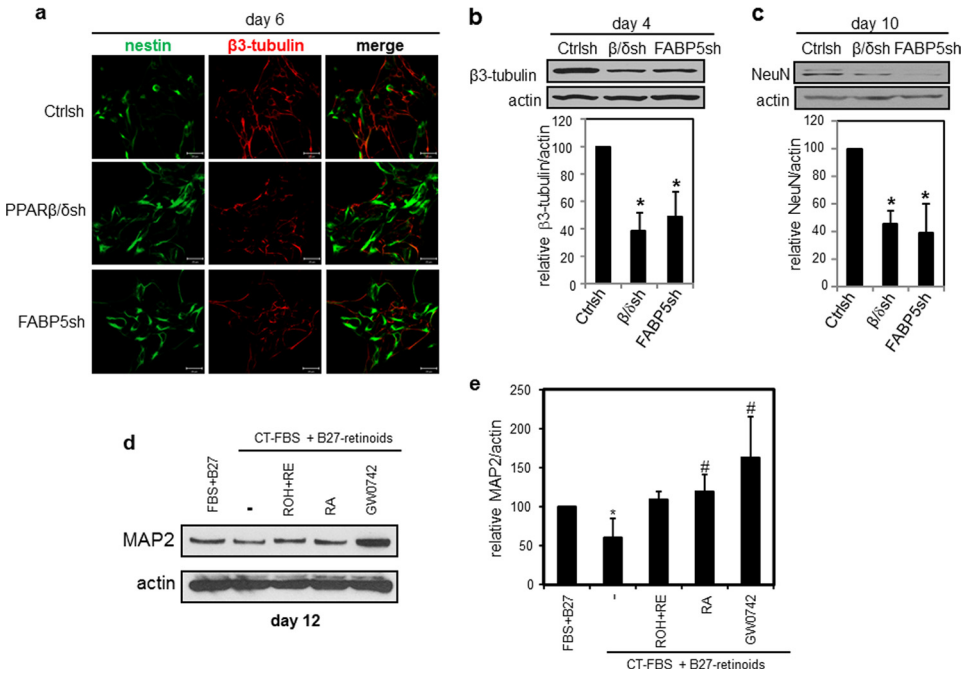

FIGURE 4. **RA signaling through the PPARβ/δ/FABP5 pathway promotes differentiation of neuronal progenitor cells to mature neurons.** P19 cells and corresponding cells with stable reduced levels of PPARβ/δ or FABP5 were induced to differentiate. *a,* cells were fixed at day 6, immunostained using antibodies against nestin (*green*) and β3-tubulin (*red*), and imaged by confocal fluorescence microscopy. A representative field for each cell line is shown. *Bar,* 20 μM. The experiment was independently repeated three times with similar results. *b* and *c, top panel,* expression of the neuronal markers β3-tubulin and NeuN in the cell lines was assessed by immunoblots at day 4 (*b*) and day 10 (*c*). *Bottom panel,* quantification of immunoblots from four independent experiments. Data are mean ± S.D. *, $p < 0.01$ *versus Ctrlsh* (Student's two-tail *t* test). *d,* P19 cells were induced to differentiate by addition of RA. On day 4, cells were transferred to poly-L-lysine-coated tissue plates and cultured in media containing charcoal-treated serum (*CT-FBS*) and supplemented with neurobasal medium containing B27 supplement devoid of retinoids (*B27-retinoids*), or supplemented with retinol (*ROH*, 0.35 μM) and retinyl ester retinyl acetate (*RE*, 0.3 μM), or RA (0.5 μM), or GW0742 (1 μM). The appearance of the mature neuronal markers MAP2 was assessed by immunoblots on day 12. *e,* quantitation of data as in *d* from three independent experiments. Data are means ± S.D. *, $p < 0.05$ *versus* FBS + B27 containing retinoids. #, $p < 0.05$ *versus* CT-FBS + B27-retinoids.

(29–31). The basis for the phenomenon is not well understood, although it is often ascribed to stabilization of some mRNAs by the compound. Nevertheless, the increase in expression of Ajuba and SIRT1 upon cotreatment with RA and cycloheximide significantly surpassed that observed in the presence of cycloheximide alone (Fig. 5*d*). Hence, RA-induced, PPARβ/δ-mediated up-regulation of ADFP, Ajuba, SIRT1, and PPARβ/δ did not require *de novo* protein synthesis, indicating that these genes are direct targets for the receptor in P19 cells.

Examination of the promoter of the mSIRT1 gene (NUBIScan Version 2.0) revealed the presence of a DR-1 response element (GGTCCAgAGAGCA) at −606 bp and a DR-2 sequence (ACAGGAtcACTTGG) at −1.1 kb upstream from the transcription start site. Chromatin immunoprecipitation (ChiP) assays indicated that neither PPARβ/δ nor RXR bound to the DR-1 region (data not shown), but in agreement with the reports that a DR-2 element may function as PPRE in some promoters (32–35), ChiP assays revealed that both PPARβ/δ and RXR associate with this region in P19 cells (Fig. 5, *e* and *f*). Functional PPREs, which efficiently recruit both PPARβ/δ and RXR, were also found in the promoters of the mAjuba (ACGGGGaACTGGA at −312 bp) and mPPARβ/δ (AGGTCAgAGGACA at −2.826 kb) genes (Fig. 5, *e* and *f*). In accordance, overexpression of either PPARβ/δ or FABP5 potentiated the ability of GW0742 to induce the expression of SIRT1 and Ajuba (Fig. 5*g*).

Enhancement of Neuronal Maturation by PPARβ/δ Is Mediated by PDK1—To understand the mechanism by which activation of PPARβ/δ promotes the transition of neuronal progenitors to mature neurons, we considered the well established direct PPARβ/δ target gene 3-phosphoinositide-dependent kinase-1 (PDK1) (36). This kinase was recently reported to be closely involved in neuronal differentiation both in cultured cells and *in vivo* (37, 38). The PPARβ/δ agonist GW0742 induced the expression of PDK1 (Fig. 6*a*). In agreement with the involvement of PPARβ/δ in regulating PDK1 expression, decreasing the expression of the receptor markedly reduced the expression of the kinase (Fig. 6*b*), and ectopic overexpression of either PPARβ/δ or FABP5 potentiated the ability of GW0742 to induce PDK1 (Fig. 6*c*).

Attesting to its regulation by RA, PDK1 expression during late phase differentiation was reduced upon removal of retinoids from the media and recovered by replenishing media with either RA precursors or RA itself (Fig. 6, *d* and *e*). The involvement of PDK1 in promotion of neuronal maturation by PPARβ/δ was then examined. Decreasing the expression of PPARβ/δ in P19 cells hampered neuronal maturation, reflected by lower expression of β3-tubulin. Notably, ectopic expression of PDK1 in these cells rescued the inhibition and allowed maturation to proceed (Fig. 6*f*). The data thus indicate that promotion of neuronal maturation by PPARβ/δ path is mediated, at least in part, by PDK1.

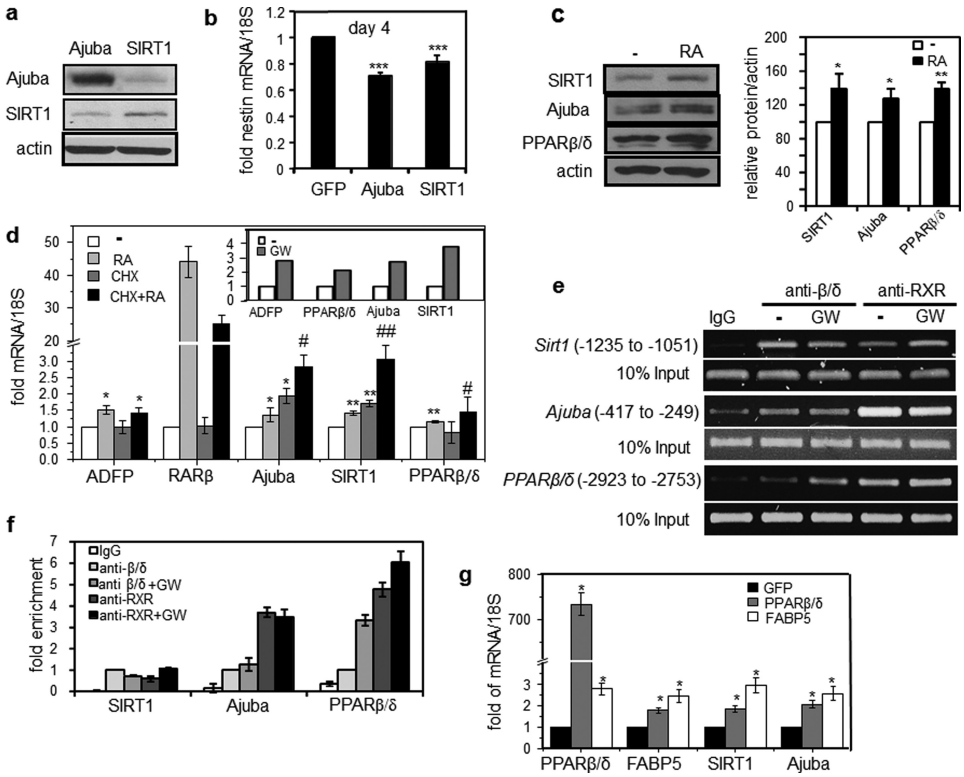

FIGURE 5. **SIRT1 and Ajuba mediate inhibition of P19 cell differentiation to neuronal progenitors by the PPARβ/δ/FABP5 pathway.** *a,* immunoblots demonstrating overexpression of SIRT1 or Ajuba in P19 cells. *b,* cells ectopically expressing GFP, Ajuba, or SIRT1 were induced to differentiate, and nestin expression was accessed by Q-PCR at day 4. Data are means ± S.D. (four independent experiments). ***, $p < 0.001$ *versus* GFP-transfected cells. *c,* P19 cells were treated with RA (1 μM). *Left panel,* immunoblots of denoted proteins 24 h post-treatment. *Right panel,* quantification of immunoblots from three independent experiments. Data are means ± S.D. *, $p < 0.05$; **, $p < 0.01$ *versus* untreated cells. *d,* cells were pretreated with vehicle or cycloheximide (*CHX*) (10 mM, 30 min) and then treated with RA (1 μM, 4 h). Levels of mRNAs were measured by Q-PCR. Data are mean ± S.D. (three independent experiments). *, $p < 0.05$; **, $p < 0.01$ *versus* nontreated controls; #, $p < 0.05$; ##, $p < 0.01$ *versus* cycloheximide alone. *Inset,* cells were pretreated with cycloheximide (10 mM, 30 min) and then treated with GW0742 (1 μM, 4 h). Levels of mRNAs were measured by Q-PCR. Data from a representative experiment are shown. The experiment was carried out twice with similar results. *e,* P19 cells were treated with vehicle or GW0742 (1 μM, 1 h). ChIP assays were performed as described under "Experimental Procedures." Rabbit IgG was used as control. *f,* quantitation of band intensities in *e. g,* cells stably overexpressing GFP, PPARβ/δ, or FABP5 were cultured in charcoal-treated media overnight and then treated with GW0742 (20 nM, 4 h). Levels of mRNAs were measured by Q-PCR. Data are means ± S.D. (three independent experiments). *, $p < 0.001$ *versus* GFP-expressing cells.

Ablation of FABP5 Results in Excess Neuronal Progenitor Cells and in Deficit in Mature Neurons in Vivo—The localization of FABP5 in C57BL/6 mouse brain was examined by immunofluorescence microscopy (Fig. 7*a*) and by immunoblots of extracts of dissected brain regions (Fig. 7*b*). The protein was found to be expressed in the hippocampus, thalamus, hypothalamus, cerebral cortex, and brain stem and to display low expression in the olfactory bulb and cerebellum. Hippocampi of WT and FABP5$^{-/-}$ mice were then compared to examine whether the lack of the binding protein affects neuronal differentiation *in vivo*. In agreement with the observations that FABP5 inhibits the formation of neuronal progenitor cells in culture, hippocampi of FABP5$^{-/-}$ mice expressed higher levels of the neural progenitor markers nestin and SOX2 as compared with WT mice (Fig. 7, *c* and *d*). Furthermore, in agreement with the observations that the FABP5/PPARβ/δ path promotes neuronal maturation, histological and biochemical analyses (Fig. 7*e* and Fig. 8, *a–c*) showed that expression of the mature neuronal markers MAP2 and NeuN are significantly lower in hippocampi of FABP5$^{-/-}$ *versus* WT mice. In contrast, expression of the non-neuronal marker glial fibrillary acidic protein is not altered in FABP5$^{-/-}$ hippocampi (Fig. 8, *a* and *d*). Hence, FABP5 inhibits progenitor formation and enhances neuronal maturation *in vivo*. Hippocampi of FABP5$^{-/-}$ mice also showed decreased expression of PDK1 (Fig. 8, *a, e,* and *f*), further supporting the identification of *PDK1* as a FABP5/PPARβ/δ target gene in neurons.

DISCUSSION

RA potently induces neuronal differentiation, but the mechanism through which it exerts this activity and the contributions of the two nuclear receptors that are activated by the hormone, RAR and PPARβ/δ, to the process are incompletely understood. The partitioning of RA between RAR and PPARβ/δ in cells is controlled by the relative expression levels of their respective cognate intracellular lipid-binding proteins, CRABP-II and FABP5. Consequently, multiple biological activities of RA critically depend on maintaining a proper balance between these intracellular lipid-binding proteins. For example, it was reported that shifts in RA signaling, brought about by alterations in the CRABP-II/FABP5 ratio, underlie the ability of the hormone to inhibit the growth of some cells while enhancing proliferation and survival in others (7, 8). It was also demonstrated that a shift in the CRABP-II/FABP5 ratio is critical for regulation of adipocyte differentiation by RA (6, 39) and that interplay between its two paths underlie the ability of RA to

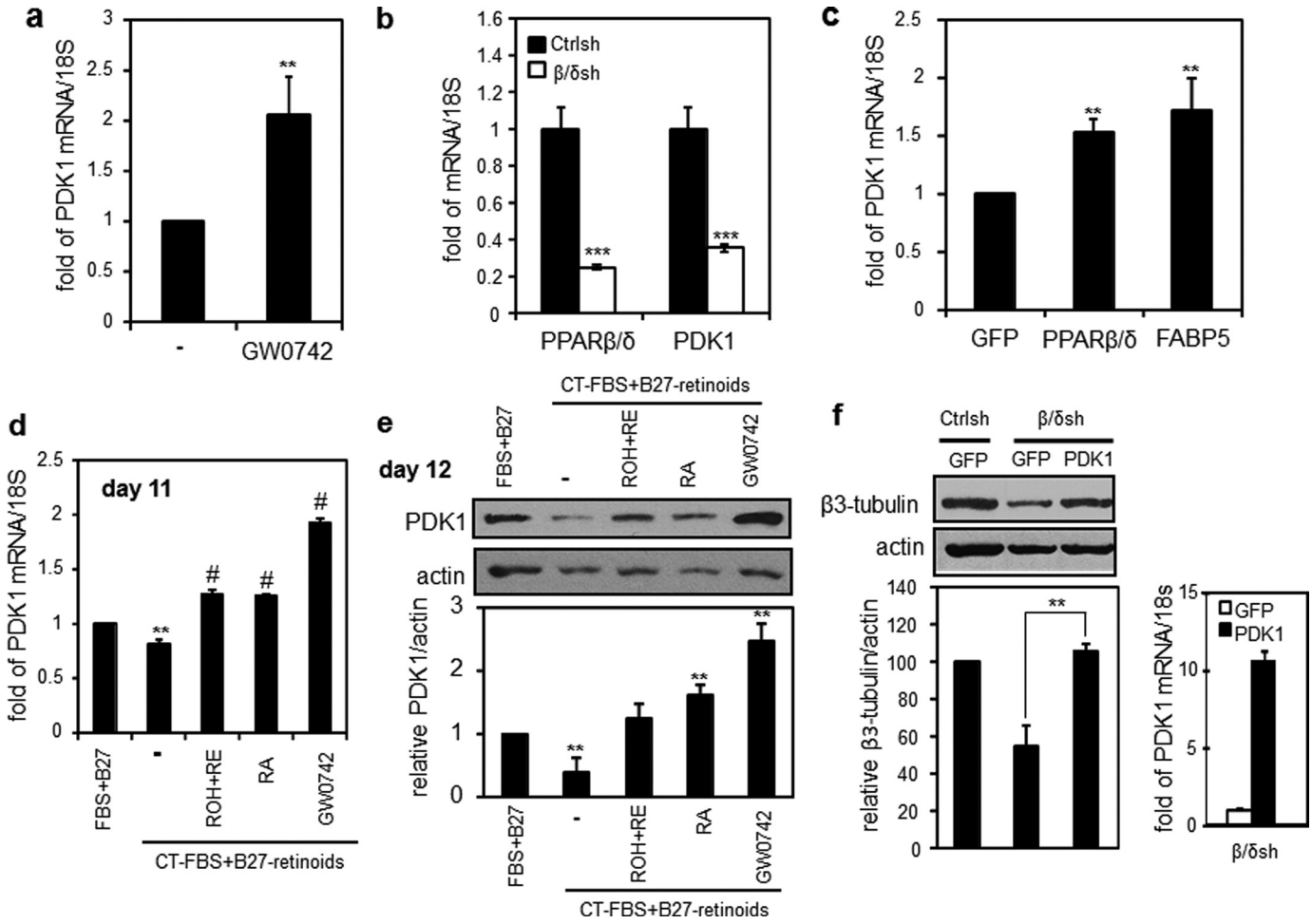

FIGURE 6. **PDK1 mediates promotion of progenitor cells to mature neurons by the PPARβ/δ/FABP5 pathway.** *a,* P19 cells were treated with GW0742 (20 nM, 4 h) and PDK1 mRNA assessed by Q-PCR. **, $p < 0.01$ *versus* untreated control. *b,* levels of PDK1 mRNA in cells stably expressing control shRNA or PPARβ/δ shRNA. ***, $p < 0.001$. *c,* P19 cells were transfected with expression constructs for PPARβ/δ or FABP5. Empty pLVX-IRES-GFP vector was used as control. 24 h post-transfection, cells were treated with GW0742 (20 nM, 4 h) and levels of PDK1 mRNA measured by Q-PCR. **, $p < 0.01$ *versus* GFP-expressing cells. *d* and *e,* P19 cells were induced to differentiate by addition of RA. On day 4, cells were transferred to poly-L-lysine-coated tissue plates and cultured in media containing charcoal-treated serum (*CT-FBS*) and supplemented with neurobasal medium containing B27 supplement devoid of retinoids (*B27-retinoids*) or supplemented with retinol (*ROH*, 0.35 μM) and retinyl acetate (*RE*, 0.3 μM), or RA (0.5 μM), or GW0742 (1 μM). *d,* levels of PDK1 mRNA assessed by Q-PCR on day 11. **, $p = 0.003$; #, $p < 0.002$ *versus* FBS + B27. *e, top panel,* levels of PDK1 and actin proteins were assessed immunoblots on day 12. *Bottom panel,* quantitation of immunoblots from three independent experiments. **, $p < 0.002$ *versus* FBS+B27. *f, left, top panel,* immunoblots demonstrating that the appearance of β3-tubulin is suppressed in cells with reduced expression of PPARβ/δ and is rescued upon ectopic expression of PDK1. *Bottom panel,* quantitation of blots from three independent experiments. *, $p < 0.05$; **, $p < 0.01$. *Right panel,* overexpression of PDK1 in cells expressing PPARβ/δ shRNA. All data are means ± S.D. (three independent experiments). *p* values were determined by a two-tail Student's *t* test.

regulate energy homeostasis and insulin responses (5, 39). The findings of this study demonstrate that coordinated alterations in the ratio of the RA-binding proteins and the ensuing shifts in activation of RA nuclear receptors are also critical for neurogenesis.

The observations show that RA-induced neurogenesis requires contributions from both the CRABP-II/RAR and the FABP5/PPARβ/δ paths, but that the two pathways are differentially employed in different stages of the process. The data indicate that early steps in RA-induced neurogenesis, entailing differentiation of stem cells into neuronal progenitors, are driven by CRABP-II and RAR and can be inhibited by the FABP5/PPARβ/δ path. The data further show that inhibition of the commitment of stem cells to the neuronal lineage by the FABP5/PPARβ/δ path is mediated by the direct PPARβ/δ target genes Ajuba and SIRT1, known to suppress the transcriptional activity of RAR (25, 26). In contrast with their inhibitory activity in this early stage, FABP5 and PPARβ/δ promote the completion of neurogenesis by supporting differentiation of neuronal progenitor cells to mature neurons. The data indicate that this activity is mediated to a large extent by PDK1, a well established direct PPARβ/δ target gene (36) previously reported to be involved in neuronal differentiation (37, 38).

The model that emerges from the observations (Fig. 9) suggests that neuronal differentiation critically relies on shifts in RA signaling that minimize activation of PPARβ/δ in early stages but allow activation of this pathway in late stages of neurogenesis. The shift is accomplished by a transient up-regulation of RARβ and CRABP-II and down-regulation of FABP5 immediately following differentiation induction. Hence, in undifferentiated P19 cells, the CRABP-II/FABP5 ratio is low, enabling RA to activate both RAR and PPARβ/δ. Following induction of differentiation, the CRABP-II/FABP5 ratio rapidly rises, effectively blocking RA signaling through PPARβ/δ and

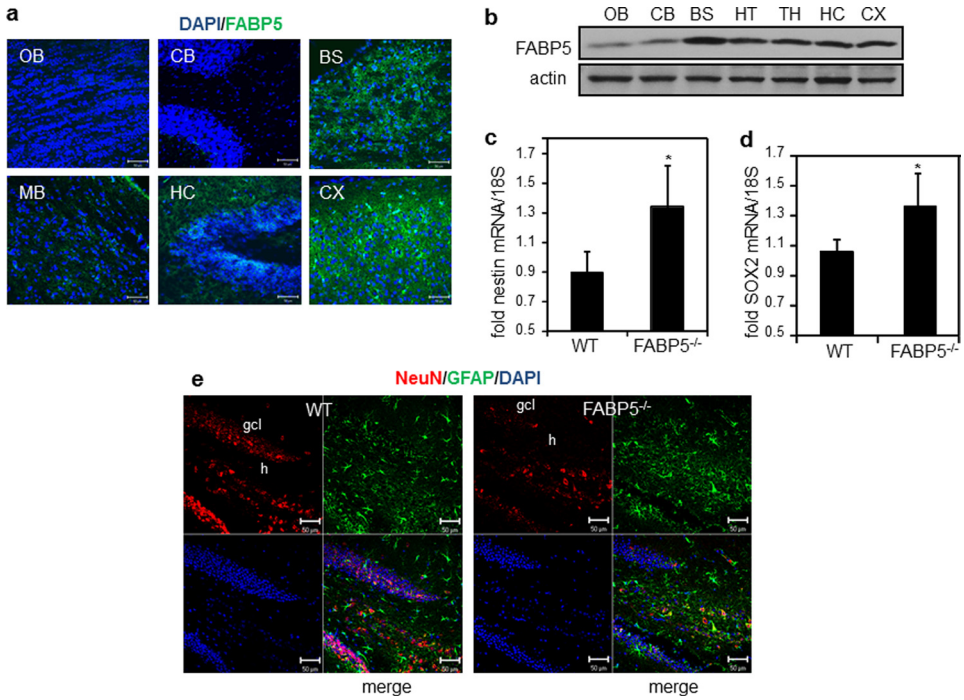

FIGURE 7. **Localization of FABP5 in mouse brain and effects of its ablation on neuronal markers in hippocampus.** *a* and *b*, location of FABP5 in mouse brain, analyzed by confocal fluorescence microscopy (*a, bar,* 50 μM) and by immunoblots (*b*). *OB,* olfactory bulb; *CB,* cerebellum; *BS,* brain stem; *HT,* hypothalamus; *TH,* thalamus; *HC,* hippocampus; *CX,* cerebral cortex; *MB*, midbrain. *c* and *d,* expression of nestin (*c*) and SOX2 (*d*) in hippocampus of WT and FABP5$^{-/-}$ mice assayed by Q-PCR. *, $p < 0.04$ *versus* WT mice. $n = 4$ mice/group. *e,* immunofluorescence microscopy showing expression of NeuN (*red*) and glial fibrillary acidic protein (*GFAP*) (*green*) in hippocampus of WT and FABP5$^{-/-}$ mice. *gcl*, granule cell layer; *h*, hippocampus hilus. *Bar,* 50 μm.

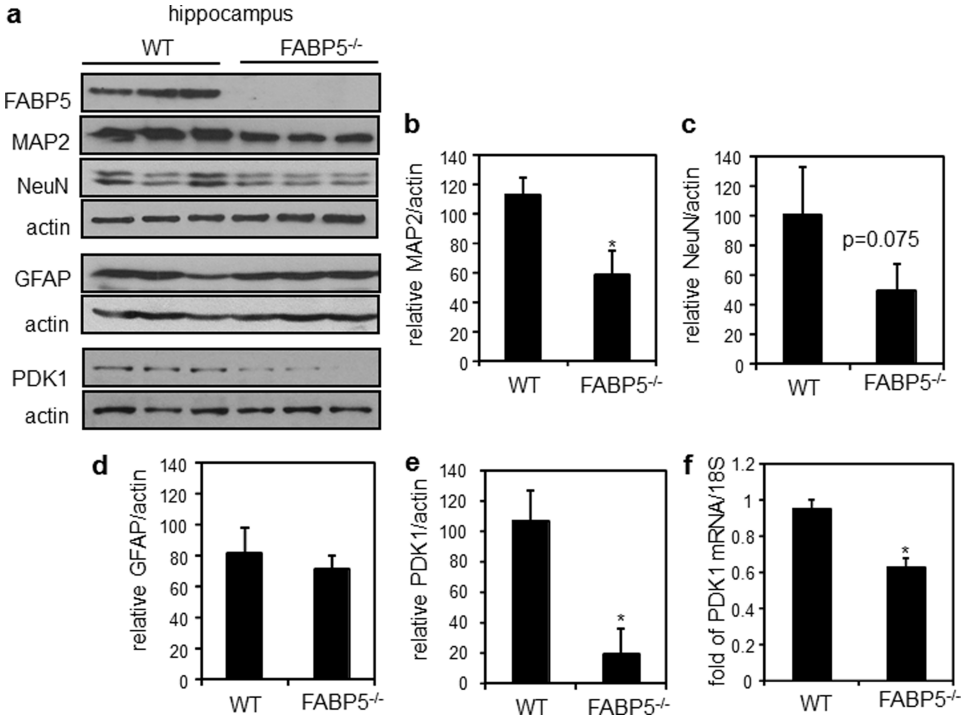

FIGURE 8. **Hippocampi of FABP5$^{-/-}$ display lower expression of mature neuronal markers.** *a,* immunoblots of denoted proteins in lysates of hippocampus of three WT and three FABP5$^{-/-}$ mice. *b–e,* quantitation of immunoblots in *a*. *, $p < 0.01$. Level of PDK1 protein (*e*) and mRNA (*f*) in hippocampus of WT and FABP5-null mice. *, $p < 0.004$. 3 mice/group. All data are mean ± S.E. *GFAP*, glial fibrillary acidic protein.

supporting exclusive activation of RAR. The increase in the CRABP-II/FABP5 ratio and in expression of RARβ is transient. It peaks 3–4 days following induction of differentiation and subsequently decreases. Consequently, RA can activate PPARβ/δ at late stages of neurogenesis, where it promotes differentiation of neuronal progenitors to mature neurons.

These studies provide important insights into the roles of FABP5 and CRABP-II and their cognate receptors in induction

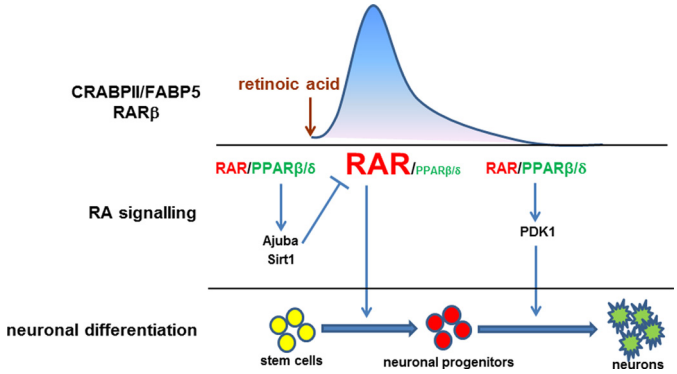

FIGURE 9. **Model for the involvement of the RA in neuronal differentiation.** RA promotes differentiation of stem cells into neural progenitor cells by activating the CRABP-II/RAR pathway. The alternative RA path, mediated by FABP5 and PPARβ/δ, can interfere with this step by inducing the expression of the RAR inhibitors Ajuba and Sirt1. This inhibition is avoided in early neurogenesis by an increase in the CRABP-II/FABP5 ratio and in the expression level of RARβ, ensuring that RA signaling is directed toward RAR. In later stages of differentiation, The CRABP-II/FABP5 ratio decreases, enabling RA to activate PPARβ/δ. In turn, the FABP5/PPARβ/δ pathway induces the expression of PDK1, thereby promoting differentiation of progenitor cells into mature neurons.

of neuronal differentiation by RA, but they did not address a potential role of the third RA-binding protein CRABP-I in the process. It has been reported in regard to this that CRABP-I expression is induced by RA in P19 cells (40). Although its function in these cells remain to be examined, the observations that CRABP-I expression in F9 teratocarcinoma cells decreases the transcriptional activity of RA (15, 16) suggest that its induction during RA-induced neuronal differentiation may serve to modulate the process.

Similarly to the localization of FABP5 in monkey brain (41), FABP5 is expressed in most regions of the mouse brain, including the hippocampus and cerebral cortex, and displays a low expression level in cerebellum and olfactory bulb. In agreement with the conclusions that the FABP5/PPARβ/δ path inhibits stem cell differentiation into neuronal progenitor cells but is necessary for differentiation of these cells to neurons, hippocampi of FABP-null mice were found to contain a higher complement of neuronal progenitor cells but fewer mature neurons. Ongoing studies aim to delineate the functional consequences of these alterations.

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